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Cytotoxic T-cell responses to HIV-1 reverse transcriptase, integrase and protease

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Objectives: To determine immunodominant regions and new epitopes for cytotoxic T cells (CTL) directed against the HIV-1 *pol* products reverse transcriptase (RT), integrase and protease in a large cohort of patients at different stages of disease.

Design and methods: Cross-sectional analysis of 98 patients from the French IMMUNOCO cohort (CD4 counts: 125–1050 × 10^6 cells/l), monitored for CTL recognition of HIV-1 *pol* products using recombinant vaccinia virus constructs and synthetic peptides.

Results: Memory CTL responses against HIV-1 *pol* products were detected in 78% of all patients whatever the stage of disease. RT was more immunogenic (81%, 30 out of 37 patients) than integrase and protease (51% and 24%, respectively). CTL recognition of RT was more frequent against Pol amino acids 310–460 (61%, 11 out of 18 patients) than against the other three portions (Pol 168–310, Pol 450–600, Pol 590–728) in patients with CD4 counts > 400 × 10^6/l, whereas in patients at advanced stages no prominent differences were observed. Two new clusters of antigenic regions were found in the NH_2 segment: three epitopes between amino-acids Pol 200 and 217 and four epitopes between amino-acids Pol 346 and 387, using five different HLA-restricting elements. A new cluster of three conserved epitopes was found in the COOH segment of RT.

Conclusions: This study shows that memory CTL responses against HIV-1 RT, integrase and protease are detectable in most patients at different stages of disease. The capacity of CTL to recognize simultaneously clusters of epitopes may become important for the immune control to reinforce antiretroviral drug efficiency.

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Keywords: AIDS, HIV, cytotoxic T cells, reverse transcriptase, integrase, protease, human T cells

Introduction

The capacity of HIV-specific cytotoxic T cells (CTL) to limit viral replication effectively is suggested by a dramatic decrease in HIV viral load following the initial appearance of CTL during primary infection and by the temporal association between high HIV-specific CTL activity and stable viral load or CD4 counts during asymptomatic stages [1–3]. Indeed, CTL can directly kill virus-infected cells or participate in the production of chemokines that can inhibit viral infection [4].

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The CTL immune responses observed during the asymptomatic stage of disease are highly polyclonal and directed against multiple antigenic regions spread all over the different HIV gene products: Env, Gag, Pol, Nef, Rev, Vif and Tat [5–9]. It has been shown in several HIV proteins [10–12] that the broad CTL clone repertoire allows also CTL recognition of different viral variants, and CTL clones can adapt to a large number of HIV epitope variants and contribute to the elimination of viral variants *in vivo* [12]. Little is known about the immunogenicity of the *pol*-encoded proteins integrase and protease [13,14], despite their important role in the virus cycle which makes them major targets for antiretroviral therapy. The recent development of efficient drugs such as protease inhibitors and their combination with reverse transcriptase (RT) inhibitors raises the question of the immunogenicity of the protease and RT. The fairly conserved *pol* gene seems one of the most interesting targets to study HIV-specific CTL, and a highly immunogenic region has been described between amino acids (aa) 359 and 383 [15], where the enzymatic function is located. Although the epitope itself shows some variation, the amino-acid exchanges that occur are often conservative: CTL recognition of this region should therefore be maintained over time. High levels of HIV-Pol specific CTL responses have been found both in patients with slow progression and low viral load and in rapid progressors [2,16]. However, the occurrence of immunodominant sites in the three *pol*-encoded proteins and their recognition at different stages of disease have not yet been systematically studied in a large number of patients.

In the present cross-sectional study we analysed memory CTL directed against the three HIV-1 *pol*-encoded proteins, RT, integrase and protease, in 98 HIV-infected patients from the French IMMUNOCO cohort. The CTL recognition of truncated HIV-1 LAI *pol* gene products and the distribution of immunodominant CTL epitopes were investigated in relation to the different stages of disease, and a number of new epitopes were delineated in the RT.

Materials and methods

Patients

The screening of CTL responses against HIV-1_{LAI} *pol*-encoded proteins was performed for 98 HLA-typed patients from the French IMMUNOCO cohort with CD4 counts of 125 to 1050 × 10^6/l. Patients at various stages of disease with different length and route of infection formed a cohort representative of the history of disease. For the present study, one blood sample per patient was taken during the first year at entry in the cohort. The patients were subdivided into three groups, according to their CD4+ count at the date of this sample:

A: above 400 × 10^6/l, n = 49 (mean CD4: 654 ± 194 × 10^6/l),

B: between 200 and 400 × 10^6/l, n = 41 (mean CD4: 299 ± 53 × 10^6/l) and

C: below 200 × 10^6/l, n = 8 (mean CD4: 160 ± 31 × 10^6/l; Table 1). The samples were randomly distributed between two laboratories and tested for CTL recognition of HIV-1_{LAI} gene products according to a standard protocol (Lab 1, Hôpital Pitié-Salpêtrière: 47 donors and Lab 2, Hôpital Cochin: 51 donors). The study was approved by the board of the Comité de Consultation pour la Protection des Personnes contre la Recherche Biomedicale of the Pitié-Salpêtrière hospital.

HLA-typing

MHC-class I types A, B and C were determined serologically by Dr D. Charron (Hôpital St. Louis) and Dr Theodorou (Hôpital Pitié-Salpêtrière), Paris, using standard microcytotoxicity assays. HLA-A2 subtyping was kindly performed by Dr Geuzoli (Hôpital St. Eloi), Montpellier and MHC class II expression was typed by Dr Theodorou, using PCR-SSP (sequence-specific primers) and sequencing of PCR products.

Recombinant vaccinia viruses

Constructs for Pol, protease, RT and integrase have already been described [14]. The construct VVTG9118 produces the protease of HIV-1_{LAI} (aa 1–167 of Pol) where the active site Asp25 Thr26 has been mutated to Lys Leu to remove the toxicity of the protease for vaccinia virus. A stop codon was introduced at position Pol 168. For the construction of VVTG8156 (Pol-1, aa 168–310), VVTG8157 (Pol-2, aa 310–460), VVTG8158 (Pol-3, aa 450–600) and VVTG8159 (Pol-4, aa 590–728), an initiation codon and a stop codon, as well as adequate restriction sites, were introduced by local mutagenesis immediately before and after the indicated positions. The truncated genes were subsequently introduced into the genome of vaccinia virus (Copenhagen strain). The construction of truncated integrase genes VVTG8161 (Pol-5, aa 728–880) and VVTG8162 (Pol-6, aa 870–1015) was done similarly.

Amplification and sequencing of HIV-1 RT proviral DNA

HIV-1 RT products were amplified from 10^6 uncultured peripheral blood mononuclear cells (PBMC) from our patients using nested PCR of proviral DNA prepared by standard phenol extraction and ethanol precipitation. Purified DNA was amplified using primers MJ3 and MJ4, as described already [17] for the first round and primers RT for [CCGGAATTCAC CATGGGTTGCACTTTAAATTTTCCCTTAGT] and RT rev [TGCTCTAGATATAGGCTGTACT GTCCATTTATCA] for the second round. PCR conditions were 2.5 mmol/l $MgCl_2$, 50 mmol/l KCl, 10 mmol/lTris–HCl pH 8.3, 200 µmol/l of each

dNTP, 0.4 μmol/l of each primer and 2.5 U Pfu *Taq*-polymerase (Stratagene Ltd., Cambridge, UK) in a total volume of 50 μl; 5 μl from the first round of amplification was used for the second PCR. Before amplification, the samples were denatured for 5 min at 94°C. Cycling parameters were 30 and 25 cycles of 1 min at 94°C, 45 s at 55°C for the first and at 50°C for the second round, 1 min 30 at 72°C for the first and second round, respectively, followed by a final elongation step of 10 min at 72°C. Consecutive cloning of PCR products was performed using the TA cloning plasmid (Invitrogen, Leek, The Netherlands), and recombinant clones were sequenced on an automated sequencer (Applied Biosystems, Foster City, California, USA) using the *Taq* cycle sequencing kit (Perkin-Elmer, Saint-Quentin, Yvelines, France) with dye primer T7 and Sp6 according to the manufacturer's instructions. Four to five clones of each sample could be sequenced. To test for rare mutations at RT 215, PCR products from primers MJ3 and MJ4 were diluted in 10-fold steps from 10^{-1} to 10^{-7} and amplified in a second round with two sets of selective primers for RT215 Y and RT215 T, respectively, as described [18]. PCR products of the second round were analysed on a 2% agarose gel.

Peptides

According to the HIV-1_{LAI} *pol* sequence [19], peptides of 25–32 aa in length were synthesized for first screening and specific restimulation of CTL. Shorter peptides were chosen according to known HLA anchor motifs for the patients' HLA molecules [20].

Peptides were either provided by the Agence Nationale de Recherche contre le SIDA (ANRS) and prepared by Neosystems, Strasbourg, France, or provided by Dr J. Berzofsky, National Institutes of Health, Bethesda, Maryland, USA, or synthesized using Fmoc chemistry by solid-phase technique on an Applied Biosystems synthesizer 430A as described [21, 22] and analyzed by reversed-phase HPLC (Beckman System Gold, Palo Alto, California, USA) and electrospray-mass spectrometry. Lyophilized peptides were dissolved in H_2O and stored at −20°C.

Antigen-presenting cells and generation of CTL lines

To analyse Pol protein recognition after viral restimulation, polyclonal HIV-specific CTL lines were generated by cocultures of patients' PBMC and autologous, irradiated phytohaemagglutinin (PHA)-stimulated cells and recombinant IL-2 (20–30 U/ml, Boehringer Mannheim SA, Meylan, France) [12]. Briefly, PBMC stimulated with 2 μg/ml PHA and irradiated after 18 h of culture, were added to thawed PBMC (10^6/ml) at a ratio of 1:5 in 24-well culture plates (Corning Costar, Brumath, France) and tested for CTL recognition after 16–24 days.

In some cases *pol* product-specific CTL were further studied by coculturing PBMC at a ratio of 5:1 with Epstein-Barr Virus (EBV)-transformed lymphoblastoid cell lines (LCL) pre-infected with recombinant HIV-1_{LAI} Pol expressing vaccinia at 5 p.f.u./cell overnight. For the inactivation of vaccinia virus constructs, (VAC, Transgene, Strasbourg, France), cells were first incubated in the dark with 5 μg/ml psoralen (trisoxsalen-4′ aminomethyl-HCl, Calbiochem, Meudon, France) dissolved in dimethyl sulphoxide at 1 mg/ml for 10 min at room temperature, exposed to a 365 nm u.v. lamp for 10 min with gentle agitation, washed three times and irradiated at 10^4 Rad [23].

Peptide-specific CTL lines were generated by coculturing PBMC with irradiated autologous PHA-stimulated cells or with EBV-transformed LCL that were preincubated for 2 h at 37°C with 1–20 μm synthetic peptides. The protein- or peptide-specific CTL lines were restimulated each 8–10 days under the same conditions. They were assayed for CTL activity on targets matched for one MHC class I or II molecule (LCL) after 3 weeks of culture and then 6–8 days after specific restimulations. HLA class-I restriction was analysed by testing CTL reactivity against two or three different HLA-matched antigen-expressing target cells and against HLA-mismatched control targets.

CTL assay

CTL activity was tested using a standard ^{51}Cr release assay as described [9]. As target cells we used autologous or HLA-matched EBV-transformed B cell lines (LCL). For recognition of HIV gene products, targets were infected with 5 p.f.u./cell recombinant vaccinia virus constructs, 18 h before CTL assays. Alternatively, PHA-stimulated, HLA-matched PBMC were used as target cells. Briefly, target cells were labeled for 2 h with 3.7×10^3 Bq $Na_2{}^{51}CrO_4$ (Amersham, Les Ulis, France). For peptide-specific recognition, target cells were incubated with the appropriate peptide (1 μm, 10 μm or serial dilutions down to 1 pm) for 2 h, before adding $3–5 \times 10^3$ labeled cells to serial dilutions of effector cells. Specific lysis was calculated as follows:

100 × (experimental − spontaneous release)/(maximal release in HCl − spontaneous release).

Spontaneous release varied between 10 and 25% of maximal release for peptide-specific and up to 35% for vaccinia virus-infected target cells.

CTL responses were considered positive when the specific response exceeded the nonspecific response by 10% or more at two effector/target (E/T) ratios or more, in at least two repeated experiments.

Statistical analysis

Statistical comparison of CTL recognition between the different groups of patients was made using the χ^2 test

and the Fisher's exact test. On the basis of unrelated cellular events, the occurrence of CTL frequencies against individual proteins and regions was also tested for statistical significance. Distribution of CTL frequencies in the different groups was evaluated using the χ^2 test with three degrees of freedom.

Results

CTL recognition of the HIV-1 *pol* gene products RT, integrase and protease

All patients' PBMC samples in this cross-sectional study were tested for CTL recognition of HIV-1 LAI Pol (Table 1) after random distribution in two laboratories using restimulation with autologous PHA blasts and IL-2 according to a standard protocol. On entry in the cohort, CTL recognition of HIV-1 Pol was found in 78% of the patients (77 out of 98) with a remarkable similarity in frequencies of CTL detection with 81%, 73% and 87% in groups A, B and C, respectively, as well as in the intensity of CTL activity (data not shown). The CTL recognition of all three *pol*-encoded proteins RT, integrase and protease was simultaneously investigated in laboratory 2 in the subgroup of 37 patients in whom Pol-specific CTL responses were detected and samples were still available (Table 1). Almost all patients tested (91%, 34/37) recognized at least one of the proteins. CTL reactivity against the three proteins was found as follows: RT was recognized with the highest frequency (in 81% of cases, 30/37), integrase with an intermediate frequency (51%, 19/37), and protease with the lowest frequency (24%, 9/37). The distribution of this CTL recognition was statistically significant ($P < 0.001$) in both groups of patients (A and B+C, Table 1), representing different stages of disease.

Definition of immunodominant regions of HIV-1 *Pol* in RT and integrase

We then characterized the immunodominant regions for CTL recognition of HIV-1 RT and integrase using truncated recombinant vaccinia virus constructs covering the whole sequence of RT and integrase, in patients with Pol-specific CTL responses. RT was tested in laboratory 1 for 31 patients and integrase in laboratory 2 for 26 patients (Table 2). Four regions, encompassing 150 aa each, were individualized in RT vaccinia virus constructs: Pol aa 168–310 (Pol-1), 310–460 (Pol-2), 450–600 (Pol-3) and 590–728 (Pol-4) and were tested in the 31 patients in laboratory 1. The region Pol-2 was recognized with a higher frequency in group A than the other regions (61%, 11/18 patients; Table 2). In groups B and C this region was recognized with a much lower frequency: 38% of the patients (five out of nine in B and none out of four in C, $P = 0.21$). In contrast, similar frequencies of CTL recognition were observed for regions Pol-1 and Pol-3 in the different groups: 44% and 39%, respectively, in group A, and 38% and 46% respectively, in groups B and C (Pol-1: five out of nine in group B, none out of four in group C, Pol-3: four out of nine in group B and two out of four in group C). The COOH region Pol-4 was recognized with the lowest frequency (28%) in patients from group A, whereas 54% of patients in groups B and C showed CTL recognition (three out of nine in B and four out of four in C; $P = 0.14$). Interestingly, in group C, CTL specific for Pol-1 and Pol-2 could not be detected in any of the four patients tested, whereas Pol-3 and Pol-4 were recognized in two out of four and four out of four patients, respectively. No statistically significant differences, however, were noted in the CTL recognition of the four regions between groups A compared with B and C, because of the limited number of patients available in groups B and C.

Two regions of HIV-1_{LAI} integrase were analysed for specific CTL recognition in 26 patients in laboratory 2, using recombinant vaccinia virus encoding for Pol-5 (aa 728–880) and Pol-6 (aa 870–1015), as shown in Table 2. The NH_2 part of Pol-5 was more often recognized in groups B and C (73%) than in group A (40%), but without statistical significance.

Characterization of new epitopes in HIV-1 RT

In order to investigate further the CTL recognition of each of the four RT subregions, individual CTL epitopes were analysed. For that purpose we selected 14 patients in laboratory 1 from groups A, B and C according to their CTL recognition of the RT subregions. The recognition and HLA restriction of individual CTL epitopes were investigated using specific restimulations with autologous virus (i.e.,

Table 1. CTL recognition of the HIV-1_{LAI} *pol* gene protein products: reverse transcriptase (RT), integrase and protease.

Groups	CD4 counts	Pol-specific CTL*	Groups	RT	Integrase	Protease
A (n = 49)	654 ± 194	40/49 (81%)	A (n = 14)	12/14	10/14	4/14
B (n = 41)	299 ± 53	30/41 (73%)	B+C† (n = 23)	18/23	9/23	5/23
C (n = 8)	160 ± 31	7/8 (87%)				
Total (n = 98) (Lab 1+2) ‡	461 ± 237	77/98 (78%)	Total (n = 37‡) (Lab 2)	30/37 (81%)	19/37 (51%)	9/37 (24%)

*Pol-specific cytotoxic T lymphocytes (CTL) analysed in the whole cohort using recombinant vaccinia virus encoding the whole HIV-1_{LAI} *pol* gene. †Groups B (20 patients) and C (three patients) were evaluated together for recognition of the individual *pol* products; ‡Patients tested against all three *pol* gene products in Lab 2.

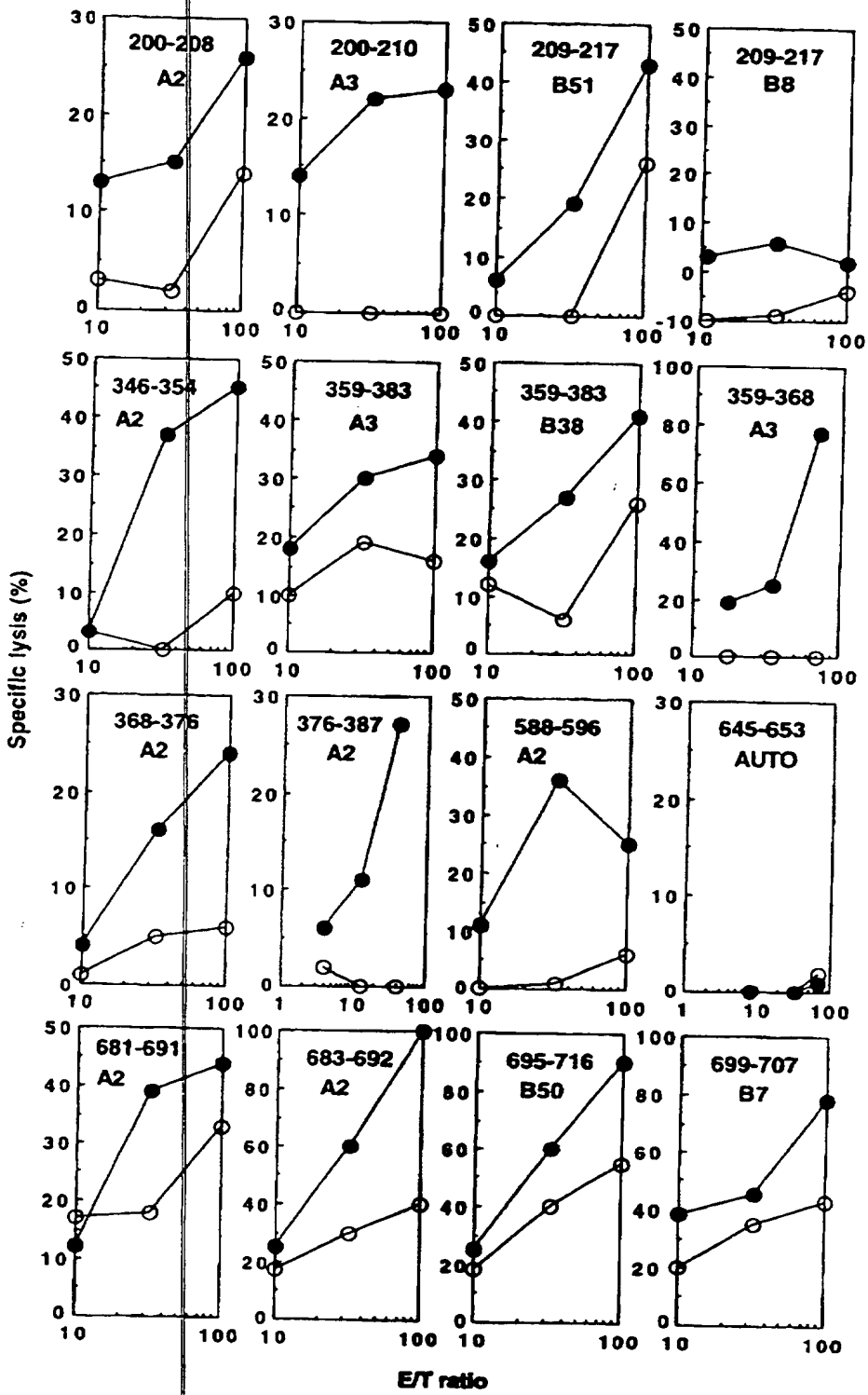

Fig. 1. Recognition of new epitopic Pol peptides all over the reverse transcriptase by specific cytotoxic T lymphocytes (CTL) from HIV-1-infected patients in the context of various HLA molecules. ●, CTL recognition of HLA-matched targets incubated with the peptides indicated in the headings; ○, targets incubated without peptide. CTL lines were generated after restimulation with recombinant vaccinia virus products corresponding to the whole HIV-1_{LAI} *pol* gene or with autologous PHA blasts. Targets were B lymphoblastoid cell lines (LCL) sharing one HLA molecule with the patient as indicated and incubated with 1 or 10 μmol/l of peptide. Auto, Tested on autologous B LCL. No peptide-specific CTL recognition could be found on HLA-mismatched targets.

Table 2. Immunodominant regions in HIV-1 RT and integrase recognized by CTL.

Patients (Lab 1)	HIV-1 RT Pol-1 (168–310)	Pol-2 (310–460)	Pol-3 (450–600)	Pol-4 (590–728)	Patients (Lab 2)	HIV-1 integrase Pol-5 (728–880)	Pol-6 (870–1015)
A: CD4 > 400 (n = 18)	8/18 (44%)	11/18 (61%)	7/18 (39%)	5/18 (28%)	A: CD4 > 400 (n = 15)	6/15 (40%)	7/15 (47%)
B+C†: CD4 ≤ 400 (n = 13)	5/13 (38%)	5/13 (38%)	6/13 (46%)	7/13 (54%)	B+C†: CD4 < 400 (n = 12)	8/11 (73%)	5/11 (46%)
Total n = 31‡	13/31 (42%)	16/31 (52%)	13/31 (42%)	12/31 (39%)	Total n = 26§	16/26 (62%)	12/26 (46%)

†Groups B (n = 27) and C (n = 3) were evaluated together for cytotoxic T lymphocyte (CTL) recognition of the reverse transcriptase (RT) and integrase regions. ‡Responders to RT who were tested for the truncated regions Pol-1 to Pol-4. §Responders to integrase who were tested for the truncated regions Pol-5 and Pol-6.

autologous PHA-stimulated cells), HIV-1 *pol*-encoded recombinant vaccinia virus or synthetic peptides. First, for each of the four RT subregions, two or three peptides of 25–32 aa length were synthesized according to the known epitopic regions of HIV-1 LAI RT [15] or to include several MHC-class I anchor motifs for HLA-A1, A2, A3/A11, B7, B8, B35, B38, B51, C7 [20]. Shorter peptides of 8–12 aa length encompassing known MHC anchor motifs for the patients' HLA haplotypes were then used to determine optimal epitopes (Fig. 1). A summary of these new epitopes which were not recognized in the context of mismatched HLA molecules is presented in Table 3: Peptide titrations showed recognition of most of these epitopes in the nanomolar range (Fig. 2).

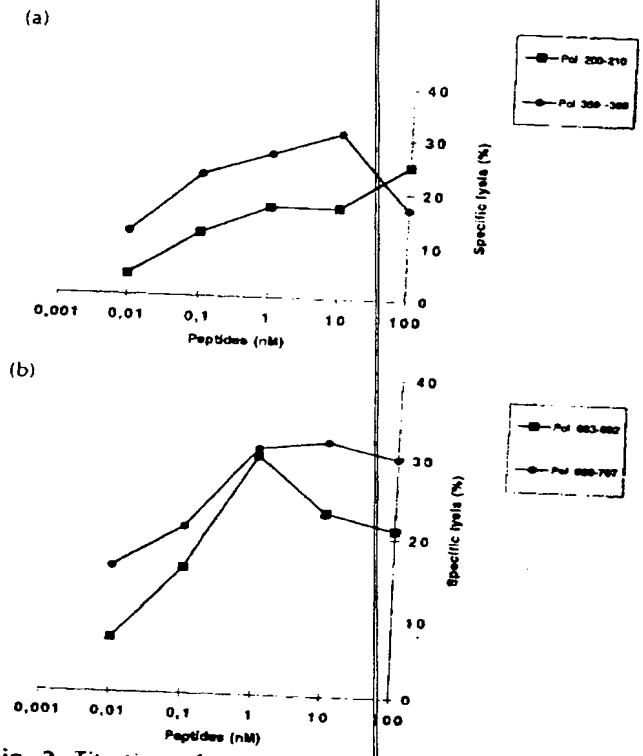

Fig. 2. Titration of peptide recognition in (a) HLA-A3-restricted cytotoxic T lymphocyte (CTL) recognition of epitopes Pol (200–210) and Pol (359–383) in the NH_2-regions of Pol-1 and Pol-2. (b) CTL recognition of epitopes Pol (683–692), restricted by HLA-A2, and Pol (699–707), restricted by HLA-B7, in the COOH region of Pol-4. CTL lines were generated after restimulation with recombinant vaccinia virus products corresponding to the whole HIV-1 LAI *pol* gene. Specific lysis is shown at effector/target (E/T) ratios of 30/1 and background lysis against targets without peptides is subtracted: (in A 4%, in B 7% for HLA-A2 targets and 2% for HLA-B7 targets without peptide).

Peptide mapping of region Pol-1 was performed after restimulation with Vac Pol-1 or with peptide Pol (187–219). We detected a cluster of new CTL epitopes: Pol (aa 200–208) was recognized in the context of HLA-A2 in three patients, whereas the overlapping epitope Pol (200–210), restricted by HLA-A3 was recognized in four patients (Fig. 2a). Recognition of Pol (209–217) in two patients was restricted by HLA-B51, but not -B8, although it contains a partial MHC class I motif with Lys at position 5 (Fig. 1).

In region Pol-2, recognized by most of group A patients, we found CTL recognition of the previously described long peptide (359–383) [15] in eight out of 10 patients with different HLA haplotypes. Specific CTL were expanded recognizing this peptide in the context of HLA-A2 (not shown), A3 or B38. We characterized two new optimal epitopes in this region, by restimulation with either peptide Pol (359–383) or Vac Pol: Pol (359–368) showing the classical MHC motif for HLA-A3/A11 was recognized in the context of HLA-A3 by four patients (Fig. 2a), whereas Pol (368–376) was recognized in three other patients. This epitope with an Ile2 and Leu9, was recognized in the context of HLA-A2. In the vicinity, two additional HLA-A2-restricted CTL epitopes were found in three patients each: Pol (346–354) and Pol (376–387). Pol (376–387) is a dodecamer and contains an Asp10 and a Lys at positions 11 and 12, rather unusual amino acids for HLA-A2 binding. In region Pol-3, we could describe one new epitope Pol (588–596) which was restricted by HLA-A2, and recognized in four patients. The COOH region Pol-4 also contains a cluster of distinct CTL epitopes. Restimulation with Vac Pol or with peptide Pol (677–709) allowed the identification of the epitope Pol (683–692; Fig. 2b) with Leu at position 2 and position 10. This epitope overlaps epitope Pol (681–691), previously described in the mouse [23].

Table 3. New cytotoxic T lympohocyte (CTL) epitopes of the HIV-1 reverse transcriptase (RT).

Pol amino acids	RT amino acids	Peptide sequence	MHC restriction element	Peptide titration*	Responding patients (n)
200–208	33–41	ALVEICTEM	HLA-A 2	0.1 nmol/l	3
200–210	33–43	ALVEICTEMEK	HLA-A 3	0.1 nmol/l	4
209–217	42–50	EKEGKISKI	HLA-B51	NT	2
346–354	179–187	VIYQYMDDL	HLA-A2	1 nmol/l	3
359–368	192–201	DLEIGQHRTK	HLA-A3	1 nmol/l	4
368–376	201–209	KIEELRQHL	HLA-A2	1 nmol/l	3
376–387	209–220	LLRWGLTTPDKK	HLA-A2	1 nmol/l	3
588–596	421–429	PLVKLWYQL	HLA-A2	0.1 nmol/l	4
683–692	516–525	ELVNQIIEQL	HLA-A2	0.1 nmol/l	3
699–707	532–540	YLAWVPAHK	HLA-B7	0.1 nmol/l	3

*Peptide concentration at which CTL lysis was higher than 50% of maximal CTL lysis. NT, Not tested.

Table 4. Cytotoxic T lymphocyte (CTL) recognition of mutations in the reverse transcriptase (RT) gene associated with drug therapy.

		CTL recognition of RT regions and epitopes					RT mutations				
		Pol 1	Pol 2		Pol 3	Pol 4					
Patients	Treatment	(RT 1–143*)	RT 143–293*	RT 209–220+	(RT 283–433*)	(RT 423–561*)	41	184	211	212	215
201.00	zidovudine	+	+	+	+	–	NM	NM	211K	212C	NM
211.00	NT	–	+	ND	–	+	NM	NM			NM
218.00	NT	+	–	ND	–	–	NM	NM			NM
220.00	zidovudine	–	–	ND	–	+	NM	NM			NM and 215Y
229.00	NT	–	–	ND	+	–	NM	NM			NM

*Length of regions determined according to the RT amino-acids. (+)Peptide 209–220 including the 215 mutation. NT, Not treated with antiretroviral drugs; ND, not done; NM, no standard drug-induced mutations.

which does not contain the classical HLA-A2 motif [20]. Both were recognized in the HLA-A2 context in three patients each (Fig. 1 and Table 3). CTL from two patients also recognized the previously described Pol (695–716) [24] in association with HLA- B50, whereas for two other patients, the restriction element could not be determined. No optimal epitope could be defined in the context of HLA-B50, but, finally, a shorter peptide from this region, Pol (699–707), was recognized in three patients, restricted by HLA-B7 (Fig. 2b). Several peptides that have been tested showed poor CTL recognition and did not completely match our criteria of CTL recognition in at least two assays at two E/T ratios and for two patients each: e.g., the already described Pol (203–219) [22]. The HLA-restricting element could not be determined for peptides Pol (193–206) and (199–223) or for peptide (652–660), which contained a HLA-A2 motif. Borderline results were also obtained with the HLA-A2 and C7 restricting elements for peptide Pol (209–217) and HLA-A3 for peptide Pol (699–707). Peptide Pol (645–653) could not be recognized at all in two patients tested despite having an HLA-A2 motif (Fig. 1).

Investigation of drug-induced mutations within HIV-1 RT epitopes

The two N-terminal RT regions Pol-1 and Pol-2 contain CTL epitopes where mutations are known to appear within the course of treatment by RT inhibitors such as zidovudine (ZDV), didanosine (ddI) or zalcitabine (ddC). According to our definition of new CTL epitopes, the most exposed epitopes for such variations are Pol 200–208 (RT 33–41), Pol 200–210 (RT 33–43), Pol 346–354 (RT 179–187), Pol 376–387 (RT 209–220) and the overlapping region Pol 359–383 (RT 192–216). We therefore determined the RT sequences and looked for the standard mutations at positions 41, 184 and 215 for five patients whose HIV-specific CTL did or did not recognize such regions. Two of five patients tested had been treated with ZDV only when tested for CTL recognition in 1991–1992. In none of the samples, however, did we find viral mutations at these very sites. Patient 201 had shown mutations at RT211 and 212, sites also present in epitopes (209–220) and (192–216). As only a limited number of sequences could be obtained, we further tested for the occurrence of the mutation RT215 by selective PCR. This method also revealed only wild-type variants in the tested patients, except for patient 220, who showed both wild-type and mutated residues at RT215. As shown in Table 4, the lack of recognition of the Pol-1 region or Pol-2 region or of the epitopes described above could not be attributed to the drug-induced mutations in these samples.

Discussion

Our study was designed to characterize memory CTL responses to the HIV-1 *pol* gene products in a representative cohort of 98 patients at different stages of disease and we detected immunodominant sites and epitopes in these proteins that may be of interest for vaccine

development. This is the first study to compare the individual CTL responses directed against protease, RT and integrase and even patients at advanced stages maintained memory CTL reactivity against HIV-1 *pol* products. Interestingly, the HIV protease is immunogenic for specific CTL, although to a lesser extent than integrase, and RT seems the most frequently recognized of the three proteins. Such differences in the immunogenicity might be explained by the relative kinetics of the synthesis and processing of the three different Pol proteins. It is possible that a higher level of RT peptides are expressed on MHC molecules at the cell surface than for the other *pol* products. Indeed, only two naturally processed peptides have been eluted from HIV-infected cells so far: one from RT (476–484) and one from Gag [25]. It has been estimated that approximately 12 molecules of the RT peptide would appear on the surface of an infected cell, a density that seems sufficient to sensitize target cells for CTL lysis. Indeed, the number of peptide-MHC complexes required for specific CTL recognition can be as low as one to three molecules, but it seems to vary depending on the effectors and targets used [25,26]. In terms of viral variation, RT and integrase seem rather conserved [19,27], whereas HIV-1 protease shows more natural polymorphism even in untreated individuals [28].

In our study, the memory CTL specific for all three *pol* products, RT, protease and integrase, remain detectable at all stages of disease, independently of the HLA haplotype, as also found for RT-specific CTL precursors in a recent study [16]. Contrasting with this maintenance of memory CTL, the loss of HIV-specifc effector CTL activity has been demonstrated in AIDS patients by our group [29], whereas other groups also showed decreases and loss in the frequencies of memory CTL with disease progression to AIDS [2,30–32,16]. Several studies using different systems to detect CTL precursors and effectors report CTL reactivity against *pol* or *gag* and *env* products at different stages of disease, but none of them showed any selective loss of recognition for those antigens [2,3,16,29–32]. In the present study, only three of the patients with CD4 counts between 125 and 200×10^6 cells/l that maintained specific CTL for HIV *pol* products had already developed AIDS. We cannot exclude the possibility that antiretroviral therapy had improved immune responses in our patients with low CD4 counts compared with the natural course of disease. However, viral loads remained high in most of the treated patients at advanced stages (data not shown), in accordance with the limited efficiency of the drugs given as mono- or bitherapy.

We showed a prominent CTL recognition of region Pol-2 in RT 310–460 in patients with high CD4 counts and dispersed recognition of all four regions in patients at advanced stages of disease, suggesting a number of CTL epitopes spread all over the RT molecule. Our study on individual RT epitopes was designed to investigate immunodominant sites for MHC-class I restricted CTL. We determined nine new epitopes that may be of potential interest for vaccine strategies. RT is known to be highly immunogenic for CTL, as initially shown by Walker *et al.* [5]. In murine models, several immunodominant T and B epitopes have been described in highly conserved regions of RT [22–24,27]. For some time, only a few human CTL epitopes have been determined in RT, and optimal MHC motifs have only recently been defined [15,22,33,20]. We were now able to delineate seven new nonamers to dodecamers in the NH2-segment of RT, six with optimal anchor residues that were recognized in the context of either HLA-A2, A3 or B51. These epitopes are contained in two clusters of antigenic regions: three epitopes in Pol-1 between aa Pol 200 and 217 and four epitopes in Pol-2 between codons Pol 346 and 387. A previously defined peptide located at the site of enzymatic activity, Pol (359–383) [15] was quite often recognized in the patients we studied, in association with HLA-A2, A3 and B38. Its length and sequence rather implied that it contained more than one epitope with optimal MHC anchor sites and two epitopes could indeed be found: Pol (359–368) and (368–376). The same region contains Pol (376–387), which does not show a classical HLA-A2 motif, although an Asp or a K at the COOH anchor have been described in HLA-A2 ligands [20,10]. Moreover, this peptide is recognized by the same number of patients as peptide Pol (346–354) which shows an optimal HLA-A2 motif. The epitope Pol (346–354) has simultaneously been investigated by Harrer *et al.* in nonprogressors [34] and overlaps another epitope described in context with HLA-A2 [16]. There are further epitopes in the vicinity at positions Pol (267–277), Pol (342–350), Pol (328–336) or Pol (370–379) [35–37,16]. The COOH epitopes in Pol-3 and Pol-4 described in this study are very conserved and were mostly defined in progressing patients. Three new CTL epitopes Pol (683–692), Pol (699–707) and Pol (695–716) have been determined, which partially overlap conserved CTL, T_H and B cell epitopes described in mice [23,24,27].

Most of our patients recognized simultaneously several newly defined and already known epitopes in the context of their different HLA molecules. However, each patient showed an individual pattern of CTL recognition and none of the epitopes was systematically recognized by all patients with the same HLA allele. For example, an HLA A2, A3 and B50 patient from group A with initially stable CD4+ counts and intermediate viral load showed CTL activity against epitopes Pol (346–354), (359–383), (368–376), (476–484), (588–596), (681–691) and (695–716). Another HLA-

A2 and -A3 patient, from group C, with low CD4+ counts and intermediate viral load only recognized epitopes Pol (346–354) and (695–716). Several epitopes are HLA-A2-restricted, most of them situated in the NH_2 part and some in the COOH segment. Three of the four patients from group C have HLA-A2 and recognize epitopes mainly located in the COOH segment, which are not necessarily also recognized by the HLA-A2 patients from the other groups. Differences in distribution of HLA types that have been associated with progression and nonprogression [38] might influence the spectrum of epitopes recognized by CTL. However, the number of patients in the different groups of this study is too small to allow a significant analysis of HLA distribution.

The fact that most immunodominant CTL epitopes have been described in conserved regions of the HIV proteins does not preclude CTL recognition of more variable epitopes, but reflects probably an experimental bias resulting from the general usage of the HIV-1-LAI sequence as a reference target antigen. The high variability of HIV protease may explain the lower CTL recognition of the HIV-1 LAI protease in our study. The differences in the level of CTL recognition of various regions of the *pol*-encoded products may also be due to mutations situated in dominant CTL epitopes [12,39,40]. Thus, the use of both variant and conserved epitopes might be important for vaccine strategies.

Interestingly, classical resistance mutations to antiretroviral drugs can occur in some of the new epitopes described here. These mutations might influence CTL recognition and therefore we checked the presence of such mutations in some patients. However, in such large studies as ours, individual viral sequences are not available for all patients. In most of the current samples of our patients, we could not detect the classical ZDV-related mutations, but only rare mutations occurring at other sites. We cannot, thus, attribute nonrecognition of epitopes to one of the classic mutations in general. Non-recognition of RT regions, in which such mutations could occur, was also observed in patients not receiving antiretroviral therapy (patients 218, 229). However, in samples from later time points, the mutation RT41 could be found for patients 201 and 220, as well as RT184 for patient 201 and RT215 for patient 220 (manuscript in preparation). There is probably a complexity of factors influencing the recognition of given epitopes or epitopic regions. On the basis of the knowledge of immunodominant regions and new CTL epitopes, current studies in our laboratory are investigating the HIV pol-specific CTL responses in individual patients over time. Our definition of clusters of new CTL epitopes should help to analyse further the consequences of antiretroviral therapy for CTL recognition.

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